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Transcription Factor Stoichiometry Drives Cell Fate: Single-Cell Proteomics to the Rescue

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In this issue of *Cell Stem Cell*, Palii et al. (2019) use a proteomics approach to investigate the differentiation along the erythroid lineage of human hematopoietic progenitors. They show that antagonistic transcription factor pairs are co-expressed in single progenitors, refuting earlier claims, and that differentiation proceeds along a continuum of cell states.

During the last few decades, the discovery that altering the stoichiometry of lineage specifying transcription factors (Ls-TFs) can induce cell fate changes has profoundly influenced our thinking about how cells differentiate. It gave rise to the concept that progenitors express balanced combinations of antagonistic Ls-TFs ("lineage priming") and that a stepwise change in their level results in the simultaneous activation of a new gene expression program and the extinction of the old one. Rapid changes in the stoichiometry of these factors could be produced by stochastic fluctuations or by external signaling (Graf and Enver, 2009). However, recent single-cell and lineage tracing experiments indicate that differentiation proceeds along a continuum rather than abrupt steps. In addition, an imaging study failed to detect co-expression of a paradigmatic antagonistic Ls-TF pair, calling the above concepts into question. In this issue of *Cell Stem Cell*, the work of Brand and colleagues now comes to the rescue, showing that antagonistic TF pairs are indeed co-expressed at the protein level in single hematopoietic progenitors before full commitment (Palii et al., 2019). The study also provides fresh evidence supporting the continuous model of cell differentiation.

Palii et al. used a cell culture system in which human hematopoietic progenitors differentiate into enucleated red blood cells within about 3 weeks (Giarratana et al., 2005). The system faithfully recapitulates various known stages of erythropoiesis, including multipotent progenitors as well as several erythroid cell types. The experiment consisted of placing CD34+ cells in culture, barcoding cell samples every 2 days for up to 22 days, and analyzing them by mass cytometry (CyTOF, Cytometry by Time-Of-Flight) (Spitzer and Nolan, 2016) using antibodies to 16 TFs and 12 cell-surface markers. After measuring the molar mass of ions in single-cell droplets and de-convoluting the barcodes, they analyzed the data by cluster-based population identification (t-SNE) and SPRING algorithms (Weinreb et al., 2018), revealing 17 clusters with a similar phenotype. These could be assigned to 12 cell states corresponding to previously described stages along the path of erythroid differentiation. Minor trajectories toward the myeloid, basophil, and megakaryocytic fates were also observed. The data showed that cell-stage-specific protein levels vary widely even within individual clusters, suggesting a continuum of differentiation with no clear separation of cell populations from early hematopoietic progenitors to late erythroid cells. This finding supports the recently proposed continuum model of differentiation (Laurenti and Göttgens, 2018).

Forced expression experiments have shown that KLF1 is a driver of the erythroid fate, and FLI1 is a driver of the megakaryocytic fate, acting in a cross-antagonistic fashion (Orkin and Zon, 2008). The CyTOF approach now permitted an examination of whether KLF1 and FLI1 are co-expressed in progenitors and become selectively upregulated after commitment, as postulated by the lineage priming/cross-antagonism model. Two key observations were made. First, both TFs are indeed co-expressed in bi-potent progenitors. Second, their levels change gradually (and oppositely) as cells progress along the erythroid trajectory, with day 2 cells expressing about 3,000 protein molecules of both factors, followed by a gradual increase of KLF1 to about 16,000 molecules at day 10 and a decrease of FLI1 to undetectable levels (summarized in Figure 1A). These findings support the idea that an increase of KLF1 relative to FLI1 results in the observed erythroid versus megakaryocytic cell fate choice. Alternatively, such changes might merely reinforce lineage identity of cells fated earlier. If quantitative changes in the relative levels of the two Ls-TFs play a role in establishing cell fate, overexpression of one of the alternate Ls-TFs in progenitors should direct differentiation toward a specific lineage; if not, they might simply block differentiation or have no effect. To test this, the authors used a lentivirus vector to overexpress FLI1 in erythroid-biased megakaryocyte erythroid progenitors (E-MEPs) and observed a dramatic increase in megakaryocytic differentiation at the expense of the erythroid fate (Figure 1B). Importantly, this occurred in the majority of cells in which FLI1 was overexpressed, therefore strongly supporting the notion that alterations in the factors' stoichiometry determines lineage choice.

These observations are important because they help clarify the current controversy about the lineage priming/Ls-TF cross-antagonism model. The model is largely based on the PU.1-GATA1 paradigm, two antagonistic Ls-TFs able to induce reciprocal erythroid-myeloid cell conversions. A key assumption of the model is that both factors are co-expressed in bi-potent or multipotent

progenitors (Graf and Enver, 2009). However, performing time-lapse experiments of branching myeloid and Meg-E cells using knocked in fluorescent reporters for PU.1 and GATA1, Hoppe et al. were unable to observe co-expression of the two TFs (Hoppe et al., 2016). This led them to conclude that the PU.1-GATA1 antagonism does not act as a decision-making mechanism but could rather serve as an execution and/or reinforcing mechanism that makes differentiation of already-made lineage choices irreversible. The present CyTOF study permitted a re-visiting of this question. Examining TF expression at various stages of differentiation, Palii et al. observed that, like KLF1 and FLI1 in E-MEPs, both PU.1 and GATA1 are expressed in common myeloid progenitors (CMPs) at a near stoichiometric ratio (Figure 1A). The apparent discrepancy between the two studies might be due to either the use of TFs fused to fluorescent proteins that could alter the transcription factor's stability or differences in the cell systems investigated. Here it should be noted that an additional TF pair, namely C/EBPa and FOG1, has also been implicated in the myeloid-erythroid branching (Mancini et al., 2012).

The proteomics study by Palii et al. has re-invigorated the concept of TF lineage priming/TF cross-antagonisms as a driving force in differentiation and further strengthened the continuum model of differentiation. The work also raises new questions: at the level of single cells moving in time, does differentiation proceed through a seamless succession of cell states or in small but discrete steps? Do individual cells show the expected gradual divergence in the stoichiometry of antagonistic Ls-TFs as the cells move away from the inferred point of lineage bifurcation? And, how is the activation of the new program coordinated with silencing of the old one? Further analyses of CyTOF data from cells differentiating in culture and in organisms should help to provide some of the answers.

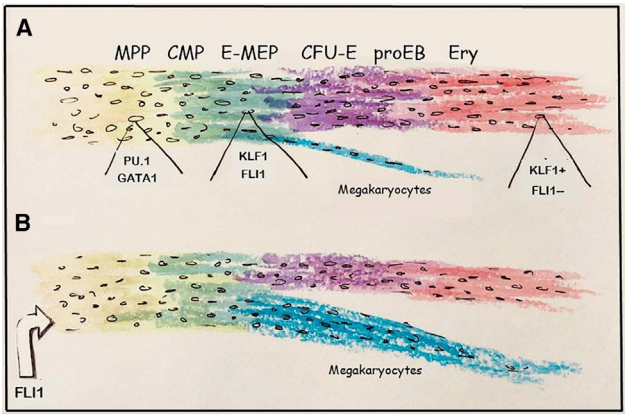

Figure 1. The Author's Representation of the Data from Palii et al.
Shown are trajectories of cells during human erythroid differentiation in culture (22 days), based on CyTOF data analyzed by t-SNE and SPRING algorithms.
(A) Differentiation of CD34+ hematopoietic/progenitor cells into erythroid cells.
(B) Differentiation of CD34+ cells infected with an FLI1 retrovirus. Some clusters with similar phenotypes identified are not shown, such as basophils and myeloid cells that emanate early. MPP, multipotent progenitor; CMP (a population known to be heterogenous; Pronk et al., 2007), common myeloid progenitor; E-MEP, erythroid-biased megakaryocyte erythroid progenitor; CFU-E, colony-forming unit erythroid (three subtypes); proEB, pro-erythroblast. Ery subsumes three more mature types of erythroblast.

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